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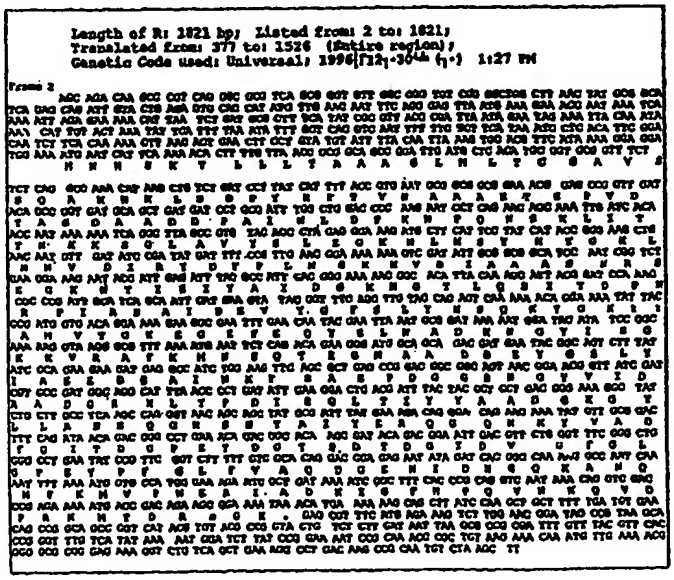
(54) Title: A STRAIN *E.COLI* JM83/pKP2 TRANSFORMED WITH A NOVEL PLASMID AND PHYTASE PRODUCED THEREFROM



(57) Abstract

This invention relates to a novel strain *E.coli* JM83/pKP2 transformed by a novel plasmid and phytase produced therefrom, and more particularly, to the strain *E.coli* JM83/pKP2 transformed with a novel recombinant vector pKP1 or pKP2, so prepared by a gene manipulation, through elucidating the gene sequence intended for the mass production of a novel phytase serving the role to enhance the phosphorous bioavailability in grains used as livestock feeds.

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A STRAIN *E.coli* JM83/pKP2 TRANSFORMED WITH A NOVEL PLASMID AND PHYTASE PRODUCED THEREFROM

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a strain *E.coli* JM83/pKP2 transformed with a novel plasmid and phytase produced therefrom and, more particularly, to a strain *E.coli* JM83/pKP2 transformed with a novel recombinant vector pKP1 or pKP2, so prepared by a gene manipulation, through elucidating the gene sequence intended for the mass production of a novel phytase serving the role to enhance the phosphorous bioavailability in grains used as livestock feeds.

Description of the Prior Art

Phytase is an enzyme which degrades phytic acid into phosphate and phosphate inositol. 50 ~ 70% of phosphate in grain used as livestock feeds exists in the form of phytic acid, but phytase is not present in monogastric animals such as hens and hogs, thus resulting in low phosphate availability. Further, indigested phytic acid phytate released to a water source has become one of the serious environment contamination sources and causes eutrophication in small lakes or tides. Further, monogastric animals can not utilize phytic acid in their intestine due to its chelation with a trace amount of minerals, amino acids and vitamins which are essential for the metabolism of livestock. These formed water-insoluble and indigestible chelate-complexes released in the form of feces are responsible for the change of the environmental ecosystem, thus inducing a serious environmental contamination.

In view of these situations, the application of phytase into the livestock feeds can reduce the supply of inorganic phosphate due to an increase of phosphate bioavailabiltity in livestock, thus leading to economic benefits. In addition, the improved availability of phosphate and other bioactive substances may also contribute

much to the reduction of the environmental contamination.

In particular, the utilization of phytase in livestock is very important in that the law regulating the amount of phosphate in animal waste was established in 1996 in Korea and, in addition to that, it has been mandatory to add phytase in the feeds of animals in the European countries. Further, when phytase is added to the feeds, it may greatly improve the productivity of livestock by enhancing the availability of some bioactive substances such as vitamins and amino acids, including some trace elements such as calcium and zinc ions whose activity is reduced by chelation with negatively charged phytate. As such, the use of feeds containing phytase in livestock can enhance the availability of feeds and reduce the environmental contamination caused by phosphate.

From the aforementioned benefits, the intensive studies with respect to phytase including the effects of phytase on animals (L.G. Young et al., 1993; X.G. Lei et al., 1994; Z. Morez et al., 1994) have been performed mainly in Europe (A.H.J. Ullah et al., 1994; K.C.Enrich, 1994; C.S. Piddington, 1993). However, since phytase can cleave a limited number of phosphate only and is mostly produced by molds which have slow growth rate, it is not economical in terms of mass production. In addition, it is difficult to use the phytic acid as an additive for monogastric animals since it is undesirable for their physiological characteristics.

The inventor, et al. have performed intensive studies for overcoming the above problems associated with phytase. As a result, a novel strain *Bacillus* sp. DS-11 producing phytase with an excellent activity and different characteristics over the conventional phytase was identified and deposited to the Korean Collection for Type Cultures within the Korea Research Institute of Bioscience and Biotechnology affiliated with the Korea Institute of Science and Technology (KCTC 0231BP), the Korean Patent Strain Depository Institute. The above patent application was filed with the Korean Industrial Patent Office (The Korean Patent Appl. No.: 96-6817). Hence, various characteristics on a novel phytase produced from the microorganism were

investigated and, as a result, the novel phytase proved to be excellent on heat and pH with better stability.

From the above results, the inventor et al. sequenced the DNA by cloning some phytase-coding gene in a strain *Bacillus* sp. DS-11 under the patent application so as to ensure the mass production of a novel phytase having the above excellent characteristics. As a result, the phytase-coding gene sequence of *Bacillus* sp. DS-11 was recognized to be a novel one, being entirely different from that of *Aspergillus awamori*(WO 94-3072A), *Aspergillus ficuum* (EP 420358, US 5436156), *Aspergillus niger*(EP 420358) and *Aspergillus terreus*(EP 684313) among the genes cloned hitherto. Thus, its accessory No. U85968(dated January 21, 1997) was given from GenBank of NCBI in the U.S.A.

Next, the inventor et al. transformed *E.coli* with the plasmid vector (pKP1 or pKP2) encoding the phytase gene of *Bacillus* sp. DS-11, and the transformed strain *E.coli* JM83/pKP2 was deposited at the Korean Collection for Type Cultures within the Korea Research Institute of Bioscience and Biotechnology affiliated with the Korea Institute of Science and Technology (KCTC 0308BP dated January 28, 1997), the Korean Patent Strain Depository Institute.

SUMMARY OF THE INVENTION

Therefore, an object of this invention is to provide a plasmid vector pKP1 and pKP2 for transformation intended for mass production of phytase, a transformed strain *E.coli* JM83/pKP2(KCTC 0308BP) herewith, and a process of mass production of phytase from said strain.

DESCRIPTION OF THE DRAWINGS

Fig. 1a shows the subcloning and mapping of pKP1 by restriction enzyme;

Fig. 1b shows the subcloning and mapping of pKP 2 by restriction enzyme;

Fig. 2 shows the base sequence and the estimated amino acid sequence of

phytase DS-11;

Fig. 3a shows the relative activity of phytase DS-11, produced from a transformed strain *E.coli* JM83/pKP2, on heat;

Fig. 3b shows the relative activity of phytase DS-11, produced from a transformed strain *E.coli* JM83/pKP2, on pH.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to a novel phytase from Bacillus sp. DS11 and characterized by DNA base sequence of the sequence table 1 or amino acid sequence of the sequence table 2.

Also, this invention includes plasmid pKP1 or pKP2 containing DNA of sequence table 1, which is ligated in such a manner and expressed in *E.coli.*

Further, this invention includes a novel strain *E.coli* JM83/pKP2 (KCTC 0308BP) transformed with plasmid pKP1 or pKP2 containing the phytase-coding gene of the sequence table 1.

This invention is explained in more detail as set forth hereunder.

According to this invention, the phytase-coding gene obtained from *Bacillus* sp. DS-11 is inserted into a plasmid pUC19 vector to prepare a novel recombinant DNA expression vector pKP1 or pKP2. After culturing *E.coli* JM-83 cloned by recombinant DNA expression vector, some colonies with effective expression potency are selected and then used for the mass production of phytase via cultivation of such colonies. Further, only pKP1 or pKP2, the recombinant DNA expression vector, is isolated from the colonies to determine its DNA sequence.

This invention is explained in more detail by the following steps.

Preparation of Novel Plasmid pKP1 and pKP2

(1) Sequencing of N-terminal amino acid

Purified phytase protein was applied to SDS-polyacrylamide gel

electrophoresis (SDS-PAGE) and transferred to PVDF membrane(Bio-Rad Lab). Then, the electroblotting was performed using 10 mM CAPS(3-cyclohexylamino-1-propanesulfonic acid) buffer solution containing 10 % methanol under pH 11.0, 4 ℃ and 400 mA for 45 hours. After cleaving the desired protein band only, it was analyzed by the Edman method using a protein/peptide sequencer [Applied Biosystems model 476A Protein/Peptide Sequencer(Applied Biosystems Ins., CA, USA)].

N-terminal amino acid sequence of purified phytase protein:

Ser-Asp-Pro-Tyr-His-Phe-Thr-Val-Asn-Ala-Ala-X-Glu-Thr-Glu

(2) Amino acid sequencing of inner peptide

Purified phytase protein was added to 70 % formic acid to 1 % (w/v) concentration, and with the addition of about 100-fold mass of CNBr, the mixture was reacted at room temperature for 24 hours. Then, 100-fold water was added to the reacting solution, and the reaction was discontinued. Using the same procedure as described in the above (1), electrophoresis was carried out to determine the amino acid sequence of inner peptide.

N-terminal amino acid sequence of internal protein fragments of phytase cleaved with CNBr;

Ala-X-Asp-Asp-Glu-Tyr-Gly-Ser-Ser-Leu-Tyr

(3) Preparation of oligonucleotide probe

Oligonucleotide probe was designed based on the amino acid sequence obtained in the procedure as described in the above (1) and (2), and synthesized with DNA synthesizer(Applied Biosystems ABI 380B).

With oligonucleotide, so synthesized by the above method as a primer and chromosomal DNA of DS-11 as template DNA as well as Taq DNA polymerase and dNTP in use, polymerase chain reaction(PCR) was carried out under the following conditions:

① Denaturation: 95℃ for one minute

② Annealing: 50℃ for one minute

③ Polymerization: 72℃ for one minute

④ Post-elongation: 72℃ for 7 minutes

Under the above conditions, the PCR was carried out and followed by 1.5 % agarose gel electrophoresis to obtain 600-bp PCR product. After recovering the PCR product from the gel, it was used as a probe.

Oligonucleotide probe based on N-terminal amino acid sequence;

Amino acid sequence :

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Ser-Asp-Pro-Tyr-His-Phe-Thr-Val-Asn-Ala-Ala-X-Glu-Thr-Glu

Possible combination of codons: 5' GAT - CCT - TAT - CAT - TTT 3'

C C C C C

G

A

Oligonucleotide probe based on N-terminal amino acid sequence of internal protein fragments;

Amino acid sequence :

1 2 3 4 5 6 7 8 9 10 11

Ala-X-Asp-Asp-Glu-Tyr-Gly-Ser-Ser-Leu-Tyr

Possible combination of codons: 3' CTA - CTA - CTT - ATA - CCA 5'

G G C G G

T

C

(4) Hybridization of DS-11 genomic DNA

Chromosomal DNA derived from *Bacillus* sp. DS-11 was isolated by the Marmur method(Marmur J. 1961, Mol Biol. 3, 208). To ascertain whether the oligonucleotide probe prepared by the above (3) was appropriate in the screening of genomic library, genomic DNA cleaved with several restriction enzymes was applied to agarose gel electrophoresis and then transferred to the nylon membrane. Then,

with DIG DNA labeling and detection kit (Boehringer Mannheim, Germany)] as well as 600-bp DNA fragments as a probe, so synthesized from the above (3), southern hybridization was performed. As a result, it was confirmed that when *Hin*dIII, *Cla* I and *Pst*I were applied, the gene showed a positive signal at 2.2 kb, 4 kb and 6 kb, respectively. When the genomic library of *Bacillus* sp. DS-11 was prepared, therefore, restriction enzyme *Hin*dIII was employed.

(5) Screening for the phytase-coding gene

Chromosomal DNA of Bacillus sp. DS-11 was cleaved with *Hin*dIII and then, 3-5 kb DNA fragments were screened. Such DNA fragments were also cleaved with *Hin*dIII, ligated to vector pUC19 treated with phosphatase (CIP) and introduced into the competent *E.coli* JM83. Such transformed strain was cultured in LB(Luria-Bertani) plate containing 100 ㎍/㎖ of ampicillin at 37 ℃ for 16 hours and transferred to the nylon membrane. Further, the strain was under colony hybridization with DNA oligonucleotide probe, so synthesized from the above (3), to select some colonies representing the signal. In order to identify whether phytase gene of *Bacillus* sp. DS-11 was properly introduced into the host, the phytase activity was measured by the Fiske method (Fiske C. H. and Subbarow Y. P., J.Biol. Chem. 1925, 66, 375). As a result, 2 colonies having the signal could be obtained among 10,000 colonies. They were cultured and then plasmids, 4.9-kb in size joined by 2.2-kb insert DNA, were isolated. And such plasmid was named as pKP1. In addition, it was ascertained that the pKP1 contained phytase gene properly inserted through measuring the expression potency of phytase.

(6) Mapping and subcloning using a restriction enzyme

As a result of cleaving 4.9-kb pKP1 with several restriction enzymes, it was confirmed to be some restriction sites of *Eco*RI, *Bam*HI, *Nde*I, *Hin*cII and *Eco*RV within 2.2-kb insert DNA. To find out the genes only necessary for the expression of enzyme potency, the subcloning of the pKP1 plasmid was carried out (Fig. 1a). pKP1 and pUC19 were cleaved with *Hin*dIII and *Nde*I, respectively, joined each other.

Such plamid vector was introduced into *E.coli* JM83 so that *E.coli* JM83 with 4.4-kb pKP2 containing 1.7kb-insert DNA might be obtained(Fig. 1b).

Transformation Process of Strain

Chromosomal DNA of *Bacillus* sp. DS-11 was cleaved with *Hin*dIII and then, 3-5 kb DNA fragments was selected. Such DNA fragments were also cleaved with *Hin*dIII, ligated to vector pUC19 treated with phosphatase (CIP) to obtain a novel plasmid pKP1 or pKP2. To express such plasmid into phytase, it was introduced into the competent *E.coli* JM83 as a host. Thus, the transformed strain, was named as *E.coli* JM83/pKP2 and deposited to the Korean Collection for Type Cultures within the Korea Research Institute of Bioscience and Biotechnology affiliated to the Korea Institute of Science and Technology dated January 28, 1997 (the accession No.: KCTC 0308BP).

The bacteriological, cultural and microbiological characteristics of the transformed strain were studied, and all results were the same as that of *E.coli* except for the production capability of phytase.

Isolation and Purification of Phytase Produced from the Transformed Strain

The novel strain *E.coli* JM83/pKP2, so transformed, was cultured in LB liquid medium containing 100 μg/㎖ of ampicillin at 37℃, centrifuged and recovered. The recovered microorganism was dissolved in the Tris buffer solution (10 mM, pH 7.0) containing 5 mM $CaCl_2$ and sonicated for 1 hour using Sonifier 450. Then, the sonicated microorganism was re-centrifuged, and its supernatants were used as crude enzyme solution. The protein saturated with 50 % acetone was isolated on Fast Protein Liquid Chromatography (FPLC consisting of open column of phenyl sepharose CL-4B and Resource S superose 12HR 10/30 column), the same enzyme as phytase produced from *Bacillus* sp. DS-11 prior to gene manipulation could be isolated.

Measurement of Phytase Potency Produced from the Transformed Strain]

(1) Measurement of phytase potency

The novel strain *E.coli* JM83/pKP2, so transformed, was cultured in LB agar (Luria-Bertani) plate containing 100 ㎍/㎖ of ampicillin at 37℃ for 16 hours and transferred to the nylon membrane. The strain was applied to colony hybridization with DNA oligonucleotide probe, so synthesized in the above (3), so as to examine the colonies representing the signal. To ascertain whether phytase-coding gene of *Bacillus sp.* DS-11 was properly introduced into *E.coli* JM83, the phytase potency was measured by the Fiske method(Fiske C. H. and Subbarow Y. P., J.Biol. Chem. 1925, 66, 375). As a result, the transformed strains having complete enzymatic activity were selected.

(2) Comparison on activity and stability of phytase on heat and pH including its molecular weight

To ascertain whether phytase produced from the transformed strain *E.coli* JM83/pKP2 was the same as that phytase produced from the original strain, the activity and stability on heat and pH of phytase were compared. To measure its stability on heat, each phytase was left at predetermined temperature for 10 minutes in the same method and then its residual activity measured. As shown in Fig. 3a, when calcium ion (Ca^{2+}) was not added into the phytase-containing solution, the activity of phytase began to reduce at 40℃, while in case of adding 5 mM calcium ion, it was stabilized up to 70℃ and its activity was maintained by 50% even at 90℃.

Also, Fig. 3b shows the phytase activity depends on pH and the optimum pH of both phytases is 7.0. Further, to identify its stability on pH, each phytase was left at different values of pH for 1 hour and followed to measure its residual activity, respectively. Even at acidic condition of less than pH 4, both phytases showed significant enzymatic activity and thus, it was considered that they may be stabilized in the stomach.

Besides, both phytases have the same molecular weight of 43,000 Dalton.

From the above results, it was considered that phytase produced from the transformed strains was the same as one produced from the original one (*Bacillus* sp. DS-11).

DNA Sequencing of Phytase-coding Gene

To sequencing 1.7-kb insert DNA within pKP2, after deletion subclones in several different sizes were obtained based on restriction site. The DNA fragment of the total 1.7-kb was prepared from them with PCR using forward and reverse primers. And then, the open reading frame (ORF) of phytase consisting of 1149 nucleotides (383 amino acids) was sequenced using MacMolly 3.5 program and as a result, it was ascertained that the above phytase coincided with N-terminal amino acid of phytase (15 amino acids) isolated from *Bacillus* sp. DS-11 strain (Fig. 2). Further, it was considered that this was a novel phytase, being entirely different from that produced from the conventional *Aspergillus* sp. strains. As a result of analyzing its amino acid sequence, 80% between 175 amino acids of C-terminal of this invention and gene of operon regulated by the Sporulation Regulatory Protein of *Bacillus subtilis* was coincided.

Sequence Table 1

Sequence length : 1149

Type of sequence: Nucleic acid

Number of chain: Double helix

Shape : Linear

Sequence type : Genomic DNA

Origin :

Name of species : *Bacillus* sp.

Name of strain: DS-11

Features of sequence:

Signal representing the features : CDS

Location of presence: 377..1526

Method to determine the features : E

Signal representing the features : sig peptide

Location of presence: 377..466

Method to determine the characteristics : E

Signal representing the characteristics : mat peptide

Location of presence: 467..1526

Method to determine the characteristics: E

Sequence 1

```
          10         20         30         40         50         60
ATGAATCATT CAAAAACACT TTTGTTAACC GCGGCAGCCG GATTGATGCT CACATGCGGT
GCGGTTTCTT CTCAGGCCAA ACATAAGCTG TCTGATCCTT ATCATTTTAC CGTGAATGCG
GCGGCGGAAA CGGAGCCGGT TGATACAGCC GGTGATGCAG CTGATGATCC TGCGATTTGG
CTGGACCCCA AGAATCCTCA GAACAGCAAA TTGATCACAA CCAATAAAAA ATCAGGCTTA
GCCGTGTACA GCCTAGAGGG AAAGATGCTT CATTCCTATC ATACCGGGAA GCTGAACAAT
GTTGATATCC GATATGATTT TCCGTTGAAC GGAAAAAAAG TCGATATTGC GGCGGCATCC
AATCGGTCTG AAGGAAAGAA TACCATTGAG ATTTACGCCA TTGACGGGAA AAACGGCACA
TTACAAAGCA TTACGGATCC AAACCGCCCG ATTGCATCAG CAATTGATGA AGTATACGGT
TTCAGCTTGT ACCACAGTCA AAAAACAGGA AAATATTACG CGATGGTGAC AGGAAAAGAA
GGCGAATTTG AACAATACGA ATTAAATGCG GATAAAAATG GATACATATC CGGCAAAAAG
GTAAGGGCGT TTAAAATGAA TTCTCAGACA GAAGGGATGG CAGCAGACGA TGAATACGGC
AGTCTTTATA TCGCAGAAGA AGATGAGGCC ATCTGGAAGT TCAGCGCTGA GCCGGACGGC
GGCAGTAACG GAACGGTTAT CGATCGTGCC GATGGCAGGC ATTTAACCCC TGATATTGAA
GGACTGACGA TTTACTACGC TGCTGACGGG AAAGGCTATC TGCTTGCCTC AAGCCAGGGT
AACAGCAGCT ATGCGATTTA TGAAAGACAG GGACAGAACA AATATGTTGC GGACTTTCAG
ATAACAGACG GGCCTGAAAC AGACGGCACA AGCGATACAG ACGGAATTGA CGTTCTGGGT
TTCGGGCTGG GGCCTGAATA TCCGTTCGGT CTTTTTGTCG CACAGGACGG AGAGAATATA
GATCACGGCC AAAAGGCCAA TCAAAATTTT AAAATGGTGC CATGGGAAAG AATCGCTGAT
AAAATCGGCT TTCACCCGCA GGTCAATAAA CAGGTCGACC CGAGAAAAAT GACCGACACA
AGCGGAAAAT AA
```

Sequence Table 2

Sequencing length : 383

Sequencing form : amino acid

Shape : Linear

Sequence type : protein

Sequence

1	2	3	4	5	6	7	8	9	10	
Met	Asn	His	Ser	Lys	Thr	Leu	Leu	Leu	Thr	
Ala	Ala	Ala	Gly	Leu	Met	Leu	Thr	Cys	Gly	
Ala	Val	Ser	Ser	Glu	Ala	Lys	His	Lys	Leu	
Ser	Asp	Pro	Tyr	His	Phe	Thr	Val	Asn	Ala	
Ala	Ala	Gln	Thr	Gln	Pro	Val	Asp	Thr	Ala	5
Gly	Asp	Ala	Ala	Asp	Asp	Pro	Ala	Ile	Trp	
Leu	Asp	Pro	Lys	Asn	Pro	Glu	Asn	Ser	Lys	
Leu	Ile	Thr	Thr	Asn	Lys	Lys	Ser	Gly	Leu	
Ala	Val	Tyr	Ser	Leu	Gln	Gly	Lys	Met	Leu	
His	Ser	Tyr	His	Thr	Gly	Lys	Leu	Asn	Asn	10
Val	Asp	Ile	Arg	Tyr	Asp	Phe	Pro	Leu	Asn	
Gly	Lys	Lys	Val	Asp	Ile	Ala	Ala	Ala	Ser	
Asn	Arg	Ser	Gln	Gly	Lys	Asn	Thr	Ile	Gln	
Ile	Tyr	Ala	Ile	Asp	Gly	Lys	Asn	Gly	Thr	
Leu	Glu	Ser	Ile	Thr	Asp	Pro	Asn	Arg	Pro	15
Ile	Ala	Ser	Ala	Ile	Asp	Gln	Val	Tyr	Gly	
Phe	Ser	Leu	Tyr	His	Ser	Glu	Lys	Thr	Gly	
Lys	Tyr	Try	Ala	Met	Val	Thr	Gly	Lys	Gln	
Gly	Gln	Phe	Gln	Glu	Tyr	Gln	Leu	Asn	Ala	
Asp	Lys	Asn	Gly	Tyr	Ile	Ser	Gly	Lys	Lys	20
Val	Arg	Ala	Phe	Lys	Met	Asn	Ser	Glu	Thr	
Gln	Gly	Met	Ala	Ala	Asp	Asp	Gln	Tyr	Gly	
Ser	Leu	Tyr	Ile	Ala	Gln	Gln	Asp	Gln	Ala	
Ile	Trp	Lys	Phe	Ser	Ala	Gln	Pro	Asp	Gly	
Gly	Ser	Asn	Gly	Thr	Val	Ile	Asp	Arg	Ala	25
Asp	Gly	Arg	His	Leu	Thr	Pro	Asp	Ile	Gln	
Gly	Leu	Thr	Ile	Tyr	Tyr	Ala	Ala	Asp	Gly	
Lys	Gly	Tyr	Leu	Leu	Ala	Ser	Ser	Glu	Gly	
Asn	Ser	Ser	Tyr	Ala	Ile	Tyr	Gln	Arg	Glu	
Gly	Glu	Asn	Lys	Tyr	Val	Ala	Asp	Phe	Glu	30
Ile	Thr	Asp	Gly	Phe	Gln	Thr	Asp	Gly	Thr	
Ser	Asp	Thr	Asp	Gly	Ile	Asp	Val	Leu	Gly	
Phe	Gly	Leu	Gly	Pro	Gln	Tyr	Pro	Phe	Gly	
Leu	Phe	Val	Ala	Glu	Asp	Gly	Gln	Asn	Ile	
Asp	His	Gly	Glu	Lys	Ala	Asn	Glu	Asn	Phe	35
Lys	Met	Val	Pro	Trp	Gln	Arg	Ile	Ala	Asp	
Lys	Ile	Gly	Phe	His	Pro	Glu	Val	Asn	Lys	
Glu	Val	Asp	Pro	Arg	Lys	Met	Thr	Asp	Arg	
Ser	Gly	Lys								

This invention has the advantages of economy with respect to the preparation of phytase in a large-scale since a recombinant DNA expression vector is prepared using the sequences of DNA and amino acid in such a manner as elucidated in the above and may be introduced into other living organisms having a rapid growth rate and easily regulatable to produce phytase having excellent activity and characteristics.

CLAIMS

What is claimed is :

1. A plasmid having the DNA sequence of sequence table 1

2. *E.coli* JM83/pKP2 transformed with the plasmid having the DNA sequence of sequence table 1.

3. Phytase produced from the strain *E.coli* JM83/pKP2.

4. Phytase according to claim 3, wherein said phytase is the amino acid sequence of sequence table 2.

FIG. 1a

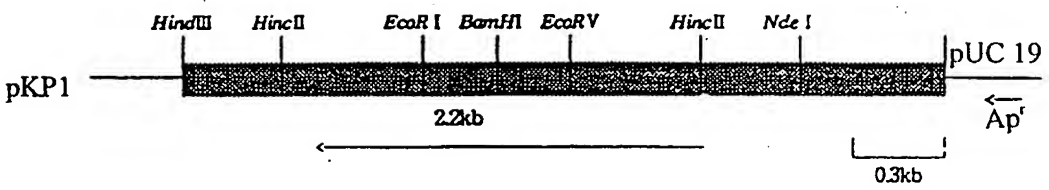
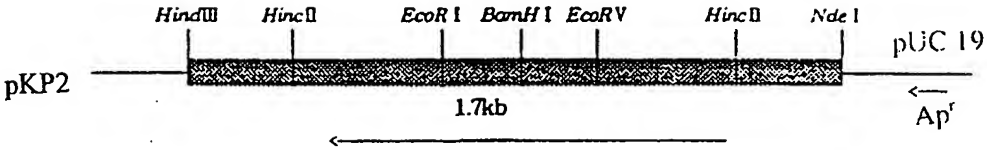

HindIII
HincII
EcoR I
BamHI
EcoRV
HincII
Nde I
pUC 19
pKP1
2.2kb
Ap^r
0.3kb

FIG. 1b


HindIII
HincII
EcoR I
BamH I
EcoRV
HincII
Nde I
pUC 19
pKP2
1.7kb
Ap^r

2/4

FIG. 2

Length of R: 1821 bp; Listed from: 2 to: 1821;
Translated from: 377 to: 1526 (Entire region);
Genetic Code used: Universal; 1996|Γ12η·30ᵘ (η·) 1:27 PM

Frame 2

```
        AGC AGA CAA GCC CGT CAG GGC GCG TCA GCG GGT GTT GGC GGG TGT CGG GGCTGG CTT AAC TAT GCG GCA
TCA GAG CAG ATT GTA CTG AGA GTG CAC CAT ATG TTG AAC AAT TTC AGC GAG TTA ATG AAA GAA ACC AAT AAA TCA
AAA ATT AGA GAA AAA CAT TAA TCT GAT GCG CTT TCA TAT CGC GTT ACC CGA TTA ATA GAA TAG AAA TTA CAA ATA
AAA CAT TGT ACT AAA TAT TCA TTT TAA ATA TTT GCT CAC GTC AAT TTT TTC TCT TCA TAA ATC CTC ACA TTC GGA
CAA TCT TCA CAA AAA CTT AAC ACT GAA CTT CCT GTA TGT ATT TTA CAA TTA AAG TGC ACG TTC ATA AAA GGA GGA
TGG AAA ATG AAT CAT TCA AAA ACA CTT TTG TTA ACC GCG GCA GCC GGA TTG ATG CTC ACA TGC GGT GCG GTT TCT
        M  N  H  S  K  T     L  L  L  T  A  A  A  G  L  M     L  T  C  G  A  V     S

TCT CAG  GCC AAA CAT AAG CTG TCT GAT CCT TAT CAT TTT ACC GTG AAT GCG GCG GCG GAA ACG  GAG CCG GTT GAT
 S  Q     A  K  H  K  L  S  D  P  Y  H  F  T  V  N  A  A  A  E  T     E  P  V  D
ACA GCC GGT GAT GCA GCT GAT GAT CCT GCG ATT TGG CTG GAC CCC AAG AAT CCT CAG AAC AGC AAA TTG ATC ACA
 T  A  G  D  A  A  D  D  P  A  I  W  L  D  P  K  N  P  Q  N  S  K  L  I  T
ACC AAT AAA AAA TCA GGC TTA GCC GTG  TAC AGC CTA GAG GGA AAG ATG CTT CAT TCC TAT CAT ACC GGG AAG CTG
 T  N  K  K  S  G  L  A  V  Y  S  L  E  G  K  M  L  H  S  Y  H  T  G  K  L
AAC AAT GTT  GAT ATC CGA TAT GAT TTT CCG TTG AAC GGA AAA AAA GTC GAT ATT GCG GCG GCA TCC  AAT CGG TCT
 N  N  V     D  I  R  Y  D  F  P  L  N  G  K  K  V  D  I  A  A  A  S     N  R  S
GAA GGA AAG AAT ACC ATT GAG ATT TAC GCC ATT GAC GGG AAA AAC GGC  ACA TTA CAA AGC ATT ACG GAT CCA AAC
 E  G  K  N  T  I  E  I  Y  A  I  D  G  K  N  G     T  L  Q  S  I     T  D  P  N
 CGC CCG ATT GCA TCA GCA ATT GAT GAA GTA  TAC GGT TTC AGC TTG TAC CAC AGT CAA AAA ACA GGA AAA TAT TAC
  R     P  I  A  S  A  I  D  E  V  Y  G  F  S  L  Y  H  S  Q  K  T  G  K  I  I
GCG ATG GTG ACA GGA AAA GAA GGC GAA TTT GAA CAA TAC GAA TTA AAT GCG GAT AAA AAT GGA TAC ATA  TCC GGC
  A  M  V  T  G  K  E  G  E  F  E  Q  Y  E  L  N  A  D  K  N  G  Y  I     S  G
AAA AAG GTA AGG GCG TTT AAA ATG AAT TCT CAG ACA GAA GGG ATG GCA GCA  GAC GAT GAA TAC GGC AGT CTT TAT
 K  K  V  R  A  F  K  M  N  S  Q  T  E  G  M  A  A     D  D  E  Y  G  S  L  Y
ATC GCA GAA GAA GAT GAG GCC ATC TGG AAG TTC AGC GCT GAG CCG GAC GGC GGC AGT AAC GGA ACG GTT ATC GAT
 I  A  E  E  D  E  A  I  W  K  F  S  A  E  P  D  G  G  S  N  G  T  V  I  D
CGT GCC GAT GGC AGG CAT TTA ACC CCT GAT ATT GAA GGA CTG ACG ATT TAC TAC GCT GCT GAC GGG AAA GGC  TAT
 R  A  D  G  R  H  L  T  P  D  I  E  G  L  T  I  Y  Y  A  A  D  G  K  G     Y
CTG CTT GCC TCA AGC CAG GGT AAC AGC AGC TAT GCG ATT TAT GAA AGA CAG GGA  CAG AAC AAA TAT GTT GCG GAC
 L  L  A  S  S  Q  G  N  S  S  Y  A  I  Y  E  R  Q  G     Q  N  K  Y  V  A  D
TTT CAG ATA ACA GAC GGG CCT GAA ACA GAC GGC ACA  AGC GAT ACA GAC GGA ATT GAC GTT CTG GGT TTC GGG CTG
 F  Q  I  T  D  G  P  E  T  D  G  T  S  D  T  D  G  I  D  V     G  F  G  L
GGG CCT GAA TAT CCG TTC  GGT CTT TTT GTC GCA CAG GAC GGA GAG AAT ATA GAT CAC GGC CAA AAG GCC AAT CAA
 G  P  E  Y  P  F     G  L  F  V  A  Q  D  G  E  N  I  D  H  G  Q  K  A  N  Q
AAT TTT AAA ATG GTG CCA TGG GAA AGA ATC GCT GAT AAA ATC GGC TTT CAC CCG CAG GTC AAT AAA CAG GTC GAC
 N  F  K  M  V  P  W  E  R  I  A  D  K  I  G  F  H  P  Q  V  N  K  Q  V  D
CCG AGA AAA ATG ACC GAC AGA AGC GGA AAA TAA ACA TGA  AAA AAG CAG CTT ATC CAA GCT GCT TTT TGA TGT GAA
 P  R  K  M  T  D  R  S  G  K  .     GAG CGT TTC ATG AGA AAG TCT TGG AAC GGA TAG CCG TAA GCA
CAG CCG GCA GCC GGT CAT ACG TGT ACG CCG GTA CTG  TCT CTT GAT AAT TAA GCG CCG CGA TTT GTT TAC GTT CAC
CCG GGT TTG TCA TAT AAA  AAT GGA TCT TAT CCG GAA AAT CCG CAA ACC CGC TGT AAG AAA CAA ATG TTG AAA ACG
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```
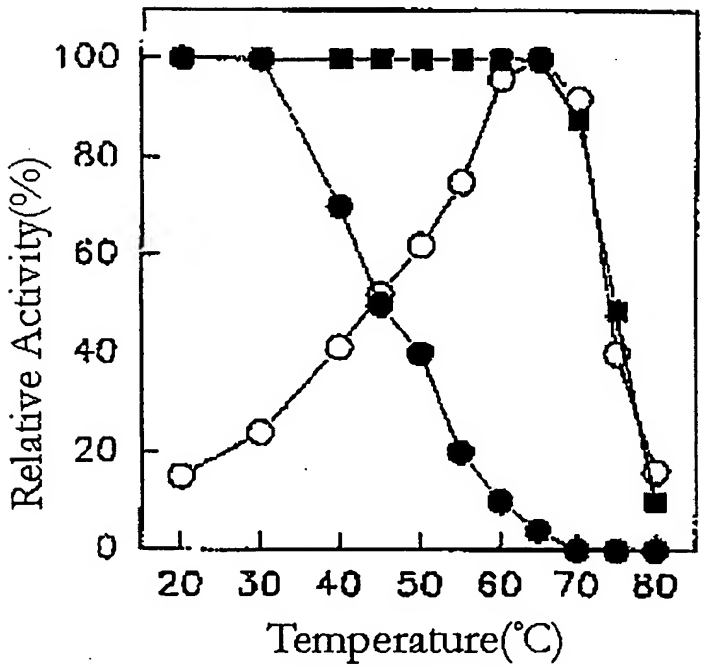

FIG. 3a

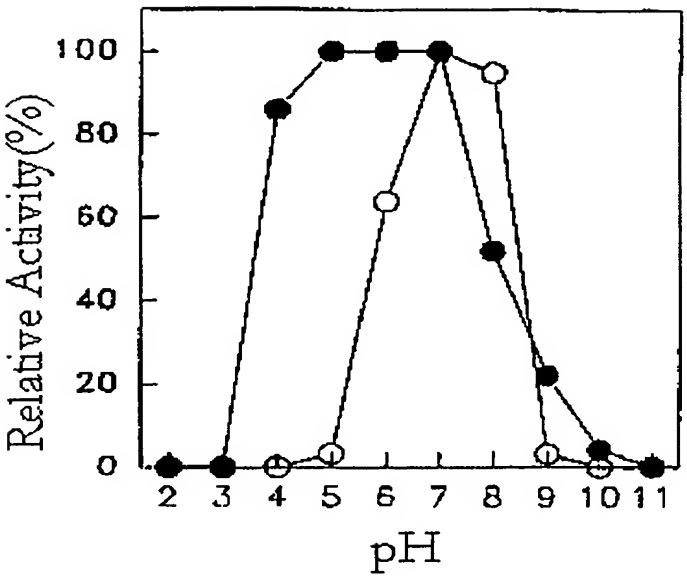


-○- Phytase produced from Bacillus sp.

-●- Phytase produced from E.coli JM83/-pKP without addition of Ca^{2+}

-■- Phytase produced from E.coli JM83/pKP2 with addition of 5mM Ca^{2+}

FIG. 3b



—○— Phytase produced from Bacillus sp.

—●— Phytase produced from JM83/pKP2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00056

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/55, 1/21, 9/16 // (C 12 N 1/21; C 12 R 1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/55, 1/21, 9/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 420 358 A1 (GIST-BROCADES N.V.) 03 April 1991 (03.04.91), claims 1,2.	3
A	EP 0 684 313 A2 (F.HOFFMANN-LA ROCHE AG) 29 November 1995 (29.11.95), claim 1.	3
A	WO 94/03 612 A1 (ALKO LTD.) 17 February 1994 (17.02.94) claims 1,2.	3

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 1998 (15.05.98)	26 May 1998 (26.05.98)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna	Wolf
Facsimile No. 1/53424/535	Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 98/00056

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
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